Cell Reports
Article

Cell PRESS
Open ACCESS

Structural and Biological Features of FOXP3 Dimerization Relevant to Regulatory T Cell Function

Xiaomin Song,[1,3,6] Bin Li,[2,3,6] Yan Xiao,[3] Chunxia Chen,[3] Qiang Wang,[3,7] Yujie Liu,[3] Alan Berezov,[3,8] Chen Xu,[4] Yayi Gao,[2] Zhiyuan Li,[2] Shiaw-Lin Wu,[5] Zheng Cai,[3] Hongtao Zhang,[3] Barry L. Karger,[5] Wayne W. Hancock,[3] Andrew D. Wells,[3] Zhaocai Zhou,[1,*] and Mark I. Greene[3,*]

[1]State Key Laboratory of Cell Biology, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China 200031
[2]Key Laboratory of Molecular Virology & Immunology, Institute Pasteur of Shanghai, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China 200031
[3]Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA
[4]Rosentiel Basic Medical Research Center, Brandeis University, Waltham, MA 02453, USA
[5]Barnett Institute, Northeastern University, Boston, MA 02115, USA
[6]These authors contributed equally to this work
[7]Present address: Women's Cancer Program at the Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA
[8]Present address: Department of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA
*Correspondence: zczhou@sibs.ac.cn (Z.Z.), greene@reo.med.upenn.edu (M.I.G.)
DOI 10.1016/j.celrep.2012.04.012

SUMMARY

FOXP3 is a key transcription factor for regulatory T cell function. We report the crystal structure of the FOXP3 coiled-coil domain, through which a loose or transient dimeric association is formed and modulated, accounting for the activity variations introduced by disease-causing mutations or posttranslational modifications. Structure-guided mutagenesis revealed that FOXP3 coiled-coil-mediated homodimerization is essential for Treg function in vitro and in vivo. In particular, we identified human FOXP3 K250 and K252 as key residues for the conformational change and stability of the FOXP3 dimer, which can be regulated by protein posttranslational modifications such as reversible lysine acetylation. These studies provide structural and mechanistic explanations for certain disease-causing mutations in the coiled-coil domain of FOXP3 that are commonly found in IPEX syndrome. Overall, the regulatory machinery involving homooligomerization, acetylation, and heteroassociation has been dissected, defining atomic insights into the biological and pathological characteristics of the FOXP3 complex.

INTRODUCTION

Regulatory T cells (Treg) play an important role in adaptive immune regulation by suppressing antiself responses of diverse immune cell populations including $CD4^+$ effector T cells, $CD8^+$ cytotoxic T cells, antigen-presenting cells, and B cells (Qin et al., 1993; Rudensky and Campbell, 2006; Sakaguchi et al., 1995, 2006; Schwartz, 2005). The forkhead/winged-helix transcriptional factor FOXP3 is selectively expressed in Tregs and functions as a dominant regulator of the development and function of this population of cells (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). Mutations of FOXP3 lead to loss of Treg suppressor function (Lin et al., 2007) and are responsible for causing "X-linked autoimmunity and allergic dysregulation" (XLAAD) syndrome or "Immunodysregulation, polyendocrinopathy and enteropathy, X-linked" (IPEX) syndrome, a fatal recessive immune disorder in humans (Bennett et al., 2001; Chatila et al., 2000; Gambineri et al., 2003; Wildin et al., 2001). In scurfy mice, a frameshift mutation in the FOXP3 gene also results in defects in T cell tolerance, characterized with excessive $CD4^+$ T cells proliferation, extensive leukocyte infiltration into certain tissues, and systemic elevation of numerous cytokines (Bennett et al., 2001; Brunkow et al., 2001; Godfrey et al., 1991; Wildin et al., 2001).

FOXP3 associates with a number of transcriptional factors including NFAT (Wu et al., 2006), NF-κB (Bettelli et al., 2005), AML1/Runx-1 (Ono et al., 2007), and RORα (Ziegler, 2006) to function as a negative regulator of gene transcription. We and others have previously characterized FOXP3 as a protein species that is part of several dynamic supramolecular complexes in which FOXP3 interacts with the histone acetyltransferase (HAT), TIP60, p300, and histone deacetylases (HDAC), HDAC7, HDAC1, and transiently with HDAC9, in Tregs (Li et al., 2007a; van Loosdregt et al., 2010; Xiao et al., 2010). FOXP3 forms a dynamic protein complex with these proteins and can differentially regulate gene transcription in a site-dependent manner. FOXP3 binding to the promoter region can lead to either histone deacetylation and transcription repression, or histone acetylation and transcription activation (Chen et al., 2006).

Dimerization of FOXP3 ensemble is required for its function as a transcriptional regulator (Chae et al., 2006; Li et al., 2007b; Lopes et al., 2006). The leucine zipper of FOXP3 is necessary and sufficient to mediate both homoassociation (Chae et al.,

2006; Li et al., 2007b; Lopes et al., 2006) and heteroassociation with FOXP1 (Wang et al., 2003). Although the forkhead domain alone may bind to DNA in vitro (Bandukwala et al., 2011; Stroud et al., 2006), the disease-associated mutations of the leucine zipper domain disrupting FOXP3 dimerization can substantially reduce the binding of FOXP3 to promoter regions in vivo (Chae et al., 2006; Li et al., 2007b; Lopes et al., 2006). Moreover, the leucine zipper is also important for the interaction between histone H1.5 and FOXP3, which cooperatively repress interleukin (IL)-2 transcription in human T cells (Mackey-Cushman et al., 2011). These findings indicate that the FOXP3 protein ensemble, as well as its DNA binding properties, could be modulated through oligomerization of the leucine zipper region. However, the molecular and atomic mechanism by which the FOXP3 dimer is regulated remains undefined.

In this study, we structurally characterized the FOXP3-Zinc finger and leucine zipper (FOXP3-ZL) domain and further revealed its functional and pathological relevance. In particular, we identified two lysine residues in the leucine zipper region as the critical sites for regulation of the FOXP3 homodimer. Alterations and modifications of these lysine residues result in changes in promoter occupancy, histone acetylation patterns, IL-2 gene expression levels, and Treg suppression activity.

RESULTS

Structure of mFOXP3 Zinc Finger and Leucine Zipper

The crystal structure of the mouse FOXP3 (mFOXP3) domain containing the zinc finger and leucine zipper region (amino acids 196–276, designated as mFOXP3 zinc finger and leucine zipper [mFOXP3-ZL]) was resolved to 2.1 Å resolution. The amino terminal region (V197-E209) corresponding to the zinc finger loop was poorly defined in the crystal structure. Based on the well-determined part corresponding to the zinc finger helix region, a complete FOXP3 zinc finger was modeled using the five-finger structure (Protein Data Bank [PDB] code 2GLI) (Pavletich and Pabo, 1993) as a homologous template.

The structure defines amino acids V197-L223 as a zinc finger motif, and D224-K262 as the leucine zipper motif (Figure 1A). The zinc finger is immediately adjacent to the leucine zipper, connecting its α helix directly to that of the leucine zipper, producing an extended single long helix. The zinc atom is coordinated by residues C198, C203, H216, H221, and in part by D220.

The zinc finger motif is not directly involved in dimerization, while the leucine zipper mediates intermolecular interactions, consistent with biochemistry showing that FOXP3 may dimerize via this region. The coiled coil has a minimum size of only four heptad repeats (Figures 1A and 1B). Its α helices wind around each other with an average pitch of 165 Å [calculated by using TWISTER (Strelkov and Burkhard, 2002)], in contrast to the 146 Å pitch observed for the prototypical coiled-coil tropomyosin structure (Brown et al., 2005). The distance between the helical axes varies by less than 0.66 Å.

Homodimerization of FOXP3 via an Unusual Antiparallel Coiled Coil

The mFOXP3-ZL homodimer features an unusual two-stranded anti-parallel α-helical coiled coil with a perfect 2-fold symmetry, resulting in two identical halves (K228-Q243 of subunit A paired with Q243-H258 of subunit B, versus Q243-H258 of subunit A paired with K228-Q243 of subunit B) (Figures 1A, S1, and S2). The two equivalent halves start respectively from the two distal ends of the elongated dimer and meet at the center core residues L241 and L245, where the 2-fold axis and the coiled-coil superhelical axis intersect. Each half contains four pairs of core residues: C231-M255 (*a*-position), Q234-L252 (*d*-position), V238-E248 (*a*-position), and L241-L245 (*d*-position). Strikingly, two out of the four pairs contain polar amino acids, which is rarely observed in coiled-coil core packing. In order to maintain the "knobs-into-holes" stacking pattern, the polar core residues Q234 and E248 turn the tips of their side chains away from the core, creating a unique conformation that favors an intersubunit hydrogen bonding essential for FOXP3 dimerization (Figures 1C and 1F).

The mFOXP3-ZL coiled coil is stabilized by a number of electrostatic interactions including four hydrogen bonds and two salt bridges. For example, the IPEX relevant residue K249 (equivalent to K250 of human FOXP3) and the breast cancer relevant residue E242 (equivalent to E243 of human FOXP3) form an intersubunit salt bridge right on the opposite side of the Q234-E248 intersubunit pairing (Figure 1D). The interface accessible surface area of mFOXP3 coiled coil is 966 $Å^2$ or 16% of total, which classifies the mFOXP3 homodimer as a transient one when compared with an average of 1,462 $Å^2$ for all determined protein dimers (1,515 $Å^2$ for homodimers and 1,335 $Å^2$ for heterodimers), and 1,005 $Å^2$ for all transient dimers (1,153 $Å^2$ for strong ones and 693 $Å^2$ for weak ones)(Reynolds et al., 2009). The empty space on the dimeric interface is ~260 $Å^3$, corresponding to substantial packing holes found near to core residues L241 and L245 (Figures 1D and 1E). These features indicate that the FOXP3 coiled coil is rather flexible and the homodimeric association is dynamic.

Sequence alignment of the coiled-coil motifs from the FOXP subfamily revealed an almost identical core, including the two polar residues at the *d*-position corresponding to mFOXP3 Q234 and E248 (Figure 1B). However, the molecular surface of the mFOXP3 homodimer shows a singularly high percentage of hydrophobic residues at *b*, *c*, and *g* positions, which is unexpected for coiled-coil structures. The three-dimensional arrangement reveals that these hydrophobic residues (specifically L222, A229, L232, L233, V237, L247, A254, and A257) form a stretch spanning the surface of the coiled coil (Figures 1B and S4).

Mutational Analysis of Interface Residues and Minimum Region for FOXP3 Homodimerization

Based on the mFOXP3 dimer structure, mutagenesis of full-length human FOXP3 was used to probe the homointeracting contribution of individual residues (Figure 1F). Of note, several IPEX mutations including L242P, DelK250, and DelE251 (equivalent to mFOXP3 L241P, DelK249, and DelE250, respectively) are found in the coiled-coil region, but with disparate three-dimensional positioning. In the mFOXP3 coiled-coil structure, L241 is a core residue (*d* position) right on the dimeric interface; K249 is an interface-flanking residue (*e* position) that forms a hydrogen bond with E242 from the opposing subunit; yet E250 is a noninterface residue sandwiched by K249 and K251 (Figures 1C–1E). Mutation or deletion of either L241 or K249 would

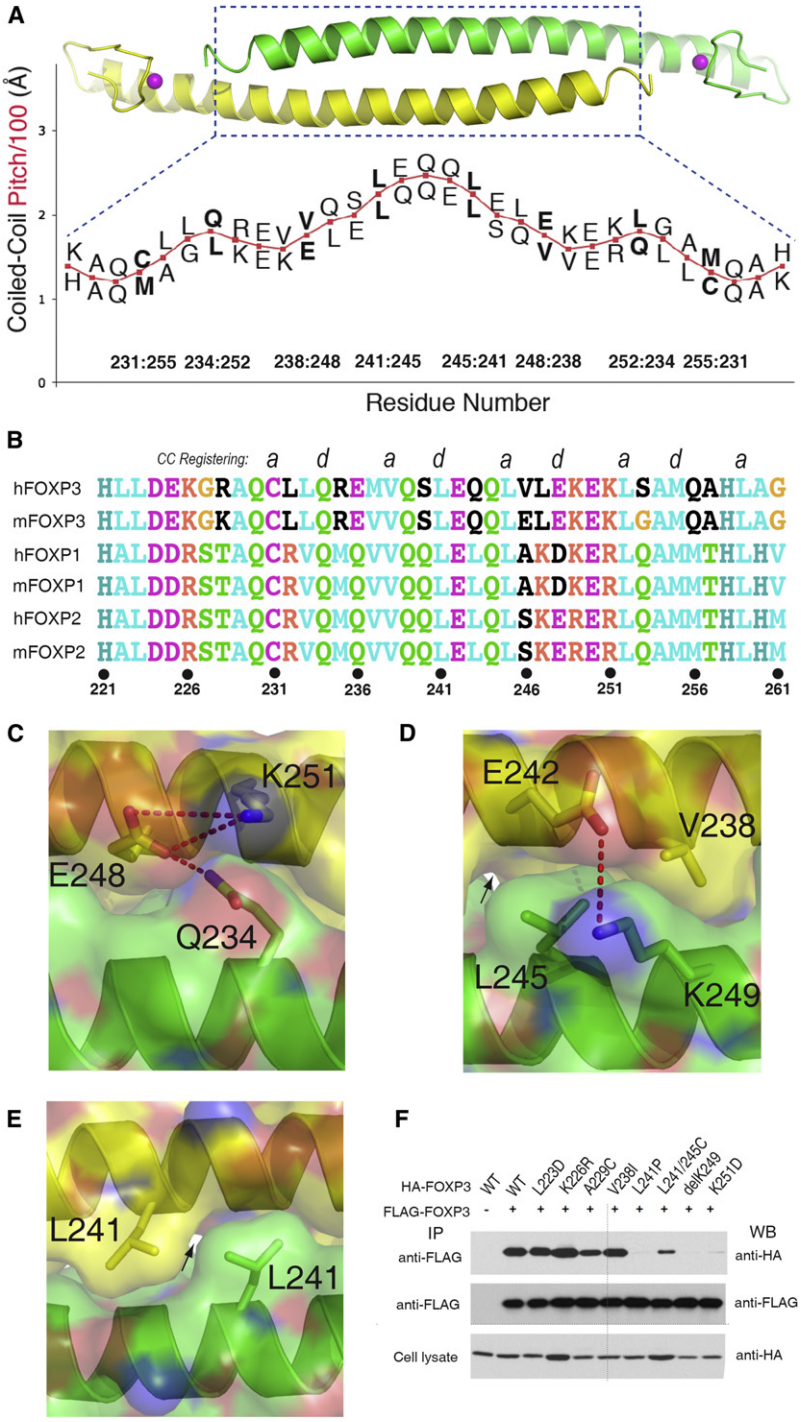

Figure 1. FOXP3 Coiled-Coil-Mediated Dimerization

(A) FOXP3-ZL forms an antiparallel dimer with a crystallographic 2-fold symmetry. Shown (top) is a structure image viewed along the 2-fold axis of mFOXP3-ZL. The coiled-coil pitch was plotted against residue number. Highlighted in bold are residues at *a* or *d* position of the coiled coil. Note that both Q234 and E248 at the coiled-coil core are nonhydrophobic residues.

(B) Protein sequence of human and mouse FOXP1, FOXP2, and FOXP3 were aligned. Coiled-coil registering and residue numbering were labeled according to the mouse FOXP3. Note that core residues Q234 and E248 forming intersubunit hydrogen bond are highly conserved, but K251 is unique for FOXP3.

(C–E) Residues of particular interest on the mFOXP3-ZL dimeric interface were highlighted. Red dash line represents salt bridge or hydrogen bond. Black arrow represents packing holes on the interface. (C) The side chain of K251 interacts with and holds up the side chain of E248 through an intrasubunit hydrogen bond. As such, the side chains of the core resides Q234 and E248 form an intersubunit hydrogen bond. (D) E242 and K249 form intersubunit hydrogen bond to stabilize the coiled coil. (E) The dimeric packing of FOXP3 at the symmetric center (around residue L241) leaves apparent hole on the interface.

(F) The homoassociation of wild-type and mutant human FOXP3 molecules by immunoblotting in the context of full-length proteins. Note that single mutant K251D disrupts FOXP3 coiled coil.

See also Figures S1 and S2.

directly affect dimerization, while deletion of E250 would alter the conformation of K249 and K251 to also affect dimerization.

We found that mutating L241 to a proline, or deletion of K249 disrupted FOXP3 homoassociation (Figure 1F). By contrast, mutation of residues L223, K226, and A229, which are not located on the dimeric interface, did not disrupt FOXP3 homoassociation. Mutation of another core residue (*a* position) V237 with an isoleucine structurally preserved the coiled-coil packing and also did not disrupt FOXP3 homoassociation.

Mutation of K251, a lysine residue involved in an interaction network essential for FOXP3 dimerization (Figure 1C), to arginine, which is found in other members of the FOXP subfamily (Figure 1B), only slightly reduced FOXP3 homoassociation (Figure 3A). K249R and K251R double mutations had little effect on dimerization. However, mutations K251Q (mimicking acetylation) and K251D reduced FOXP3 homoassociation respectively in these biochemical studies. We propose the reduction of FOXP3 homodimers occurs through modulation of the intersubunit hydrogen bond formed between Q234 and E248 (Figure 1C, 1F, and 3A). These observations indicate that the noncore residue K251, which is unique to the FOXP3 molecule, plays a role in regulating the stability of the FOXP3 homodimer.

Structure-based mutational analysis further revealed that a minimum of four haptad repeats (C_{231}LLQREV; VQSLEQQ; LELEKEK; LGAMQAH$_{258}$) is required for the FOXP3 dimer formation. Removal of the core residues (*a* or *d* position: C231 and M255) from the coiled-coil terminal haptad repeats disrupted the FOXP3 dimer as shown by the FOXP3 truncation mutant amino acids (aa) 1–254; while the aa 1–258 truncation mutant

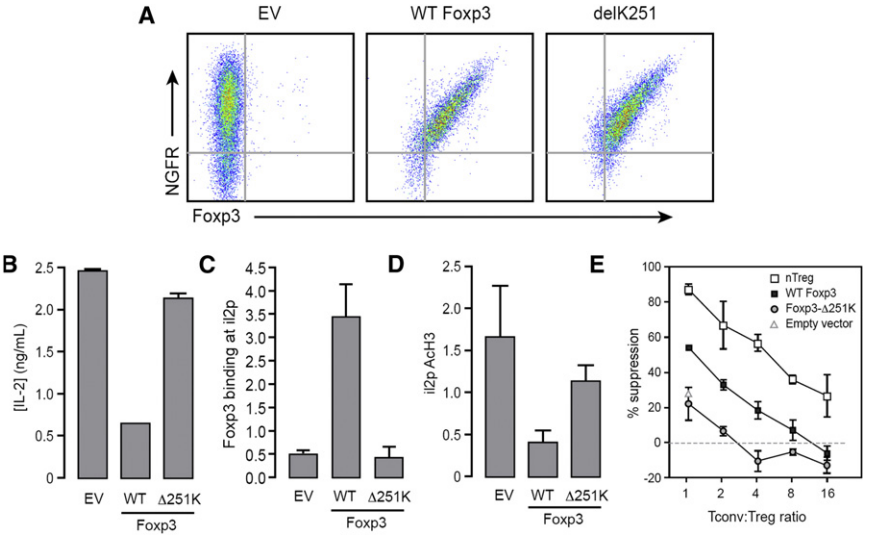

Figure 2. Lysine 251 in the Leucine Zipper Is Required for FOXP3 Function

(A) Primary murine CD4$^+$ T cells were transduced with empty MIGR1 retrovirus (left panel, EV) or MIGR1 encoding wild-type, murine FOXP3 (middle panel, WT) or the lysine 251 deletion mutant of murine FOXP3 (right panel, delK251). Greater than 95% of T cells were transduced as judged by expression of the tailless NGFR reporter, and >90% of cells transduced with FOXP3 constructs expressed FOXP3 protein.

(B–D) Cells were restimulated for 4 hr with plate-bound anti-CD3/CD28 and assessed for IL-2 secretion by ELISA (B), and FOXP3 binding (C), and histone acetylation (D) at the endogenous IL-2 promoter was assessed by ChIP analysis.

(E) CD4$^+$ T cells transduced with wild-type FOXP3 (dark gray squares), the 251K deletion mutant (light gray circles), or empty vector (open triangle) were assessed for their capacity to suppress the proliferation of naive CD4$^+$CD25$^-$ T cells in an in vitro Treg assay as compared to purified CD4$^+$CD25$^+$ Treg as a positive control (open squares). Data are depicted as the percent inhibition of CD4$^+$CD25$^-$ T cell division observed in the absence of added Treg.

can still form dimers, as does wild-type FOXP3 (Figure 3A). Structure wise, the truncation mutant aa 1–254 would also lack the intersubunit salt bridge formed by Q256 and R235, and would lose a pivotal core-packing residue M255. M255's side chain contributes to maintenance of the central conformational network formed by residues K251, Q234, and E248 (Figure 1C).

Lysine 251's Role in FOXP3 Coiled-Coil-Mediated Dimerization in Treg Cells

Since K251 has been highlighted as a unique residue critical for FOXP3 homodimerization, we then expressed wild-type mFOXP3 and mutants of mFOXP3 lacking lysine 251 (delK251) in primary murine CD4$^+$ T cells to verify its functional relevance (Figure 2A). Wild-type mFOXP3 repressed *il2* gene expression by activated T cells (Figure 2B), and this was associated with direct occupancy of mFOXP3 and deacetylation of nucleosomes at the *il2* promoter (Figures 2B and 2C). However, the delK251 mutant mFOXP3 failed to repress IL-2 production (Figure 2B), and completely failed to bind and remodel the chromatin at the endogenous *il2* promoter (Figures 2B–2D), indicating that this lysine 251 is required for proper DNA binding and transcriptional regulation in vivo. Furthermore, while CD4$^+$ T cells transduced with wild-type mFOXP3 were able to suppress the proliferation of naive T cells in an in vitro Treg assay (Figure 2E, black squares), CD4$^+$ T cells expressing the delK251 mutant mFOXP3 exhibited a nearly complete loss of suppressive function (Figure 2E, gray circles). These results emphasize the functional significance of a single residue, K251, in FOXP3 homodimerization.

Acetylation of FOXP3 Leucine Zipper and Its Structural/Functional Consequences

Mass spectrometric studies of human FOXP3 (hFOXP3) revealed that lysine residues K250 and K252 (equivalent to mFOXP3 K249 and K251, respectively) are acetylated in vivo. Notably, hFOXP3 K250 (equivalent to mFOXP3 K249) is deleted in some IPEX patients, while K252 (equivalent to mFOXP3 K251) is represented by arginine in other members of the FOXP family.

As mentioned, mFOXP3 K249 and K251 mediate a network of interactions that are critical for dimerization. Specifically, the K249 side chain forms an intersubunit salt bridge with the side chain of E242, while the side chain of K251 helps to uniquely position or "hold" the side chain of E248, a polar residue at the core (*a* position), so that it can form intersubunit hydrogen bonding with the side chain of Q234, also a polar residue at the core. The positive charges of both K249 and K251 are important for this dimer-stabilizing network. As predicted from the atomic structure, substitution of K249 and/or K251 with arginine, which maintains positive charges, would be permissive for dimerization of FOXP3 (Figure 3A). A K251D mutation that introduces negative charges disrupts FOXP3 homodimer. In addition, we found the K251D mutation also disrupts FOXP3-FOXP1 heterodimer formation (Figure 6). Acetylation of FOXP3 at K249 and/or K251 would neutralize the positive charges at these sites through which the FOXP3 dimer stability is modulated. Indeed, substitution of K251 with glutamine, which mimics acetylation, slightly reduces FOXP3 dimerization (Figure 3A).

Residues that Guide Treg Function

To explore how discrete lysines modify FOXP3 function, we transduced wild-type and mutant forms of mFOXP3 into conventional murine CD4$^+$ T cells to gauge their suppressive functions. Mutation of lysines K249 and K251 to arginines exerted little effect on FOXP3-mediated Treg function, whereas substantial impairment of FOXP3 suppressive activity occurred with mutation to either aspartic acid (D) or glutamine (Q) (Figure 3B).

Supportive data were also observed using in vivo assays of the inhibitory effects of wild-type Tregs or T cells transduced

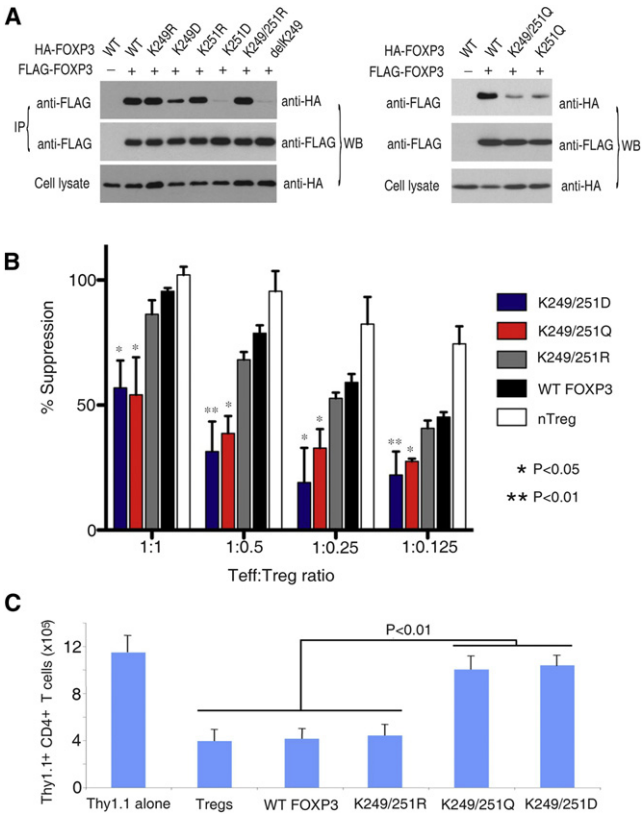

Figure 3. FOXP3 Lysine Mutations Affect Its Homoassociation and Treg Suppressive Function

(A) Homoassociation of wild-type and K249/K251 FOXP3 mutants using immunoprecipitation. The lysine residues K249 and K251, which could be acetylated in vivo were substituted with arginine, aspartic acid or glutamine to mimic the electrostatic effect introduced by posttranslational modifications. Introduction of negative charges at K251 through aspartic acid substitution disrupted FOXP3 homoassociation, while K251Q, which mimics acetylation, led to decreased binding. Removal of the core residues (*a* or *d* position: C231 and M255) from the coiled-coil terminal haptad repeats disrupted the FOXP3 dimer as shown by the FOXP3 truncation mutant aa 1–254, while the aa 1–258 truncation mutant can still form dimers.

(B) In vitro suppression assay of regulatory T cells with wild-type or lysine mutant FOXP3. To test the functional consequences of FOXP3 lysine modifications, wild-type (WT) or mutant FOXP3 was transfected into $CD4^+CD25^-$ T cells by retrovirus as described. The data shown here are representative of five separate experiments. Similar to (A), mutation of FOXP3 lysine residues K249 and K251 to aspartic acid or glutamine substantially impaired the suppressive activity of Tregs.

(C) In vivo suppression assay of regulatory T cells bearing WT or lysine mutant FOXP3. Proliferation of $Thy1.2^+$ $CD4^+CD25^-$ T cells (10^6) alone, or in the presence of either WT $Thy1.1^+$ Tregs or $Thy1.1^+$ $CD4^+CD25^-$ T cells transduced with WT or mutant FOXP3 (10^6), was assessed 7 days postintravenous injection into $RAG2^{-/-}$ mice (three mice/group); data shown are representative of three separate experiments.

with wild-type or mutant forms of mFOXP3 on the homeostatic proliferation of wild-type T cells. Transduction of T cells with either wild-type FOXP3 or mutant FOXP3 (K249/251R) led to comparable suppressive function to that of wild-type Tregs ($p > 0.05$), whereas cells bearing the aspartic acid or glutamine mutations at these residues showed impaired Treg suppression ($p < 0.01$) (Figure 3C). Since the lysines are post translationally modified, extrinsic signals appear to affect Treg cell function by charge alteration of the side chains of K249 and K251.

Alteration of the FOXP3 Acetylation Pattern by Discrete Immune Extrinsic Signals

Various histone acetyltransferases could affect discrete lysine residues of FOXP3. p300 for example appears to target residues including but not limited to K250 and K252 of human FOXP3 (equivalent to mFOXP3 K249 and K251 respectively) (Figure 4). We initially used p300 and hFOXP3 cotransfected 293T cells in Figure 4A. While lysines other than K250 or 252 may also be minimally acetylated, K250 and K252 are dominant but not the exclusive targets of the p300 HAT.

We then examined effects of extrinsic signals on this type of acetylation. $CD3^+$ Jurkat T cells that contain several endogenous histone acetyltransferases including Tip60, CBP, and p300 were transfected with wild-type or the hFOXP3 K250/K252R double-mutant constructs. The transfected Jurkat cells were then treated with transforming growth factor (TGF)-β and/or IL-6. TGF-β treatment is known to increase the level of acetylated FOXP3 leading to enhanced chromatin binding of FOXP3 (Samanta et al., 2008) and this is also shown in Figures 4B and 4D. Strikingly, TGF-β treatment led to increased acetylation not only in wild-type FOXP3, but also in K250 and K252 mutants such as K250/K252R double-mutant cells, indicating that TGF-β treatment leads to FOXP3 acetylation at other lysine residues in addition to K250 and K252.

Therefore, HATs other than p300 are likely to be induced by TGF-β and responsible for the acetylation of other sites. Interestingly, we found that iTreg inhibiting and Th17 polarizing signals, for example, TGF-β in combination with IL-6, limited acetylation of both wild-type FOXP3 and the K250/K252R double mutant in transfected Jurkat cells (Figure 4C). Furthermore, deletion of K250, which would affect FOXP3 function as shown in Figure 4, does not appear to alter the overall acetylation pattern induced by TGF-β. Taken together, these data indicate that even though FOXP3 acetylation is positively correlated with its activity as thought, TGF-β does not promote FOXP3 acetylation at sites K250 and K252, which are acetylated by p300. TGF-β promotes FOXP3 acetylation through residues other than K250 and K252, and acetylation of K250 and K252 is induced by extrinsic signals other than TGF-β.

Homoclustering of FOXP3 Dimers

The molecular surface of the FOXP3 coiled-coil dimer is highly hydrophobic when judged by its unusual surface composition: 48% nonpolar residues, in contrast with an average of 39% for general protein dimers (Reynolds et al., 2009). Consistently, crystal packing analysis revealed that the mFOXP3-ZL coiled-coil dimer tends to form clusters by bundling up multiple α helices from distinct dimers (Figures 5A and S4). The extended hydrophobic stretch on the coiled-coil surface facilitates this type of high-order packing between dimers.

There are also a number of hydrophobic and electrostatic contacts between dimers (Figure 5A). The two neighboring α helices from the two dimers pack symmetrically in an

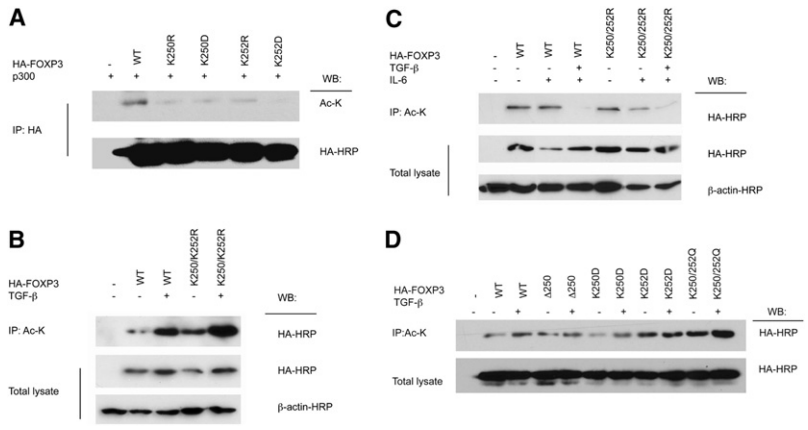

Figure 4. TGF-β Promotes FOXP3 Acetylation through Residues Other Than K250 and K252 that Were Acetylated by p300

(A) K250 and K252 are acetylated by p300. 293T cells were cotransfected with p300 and wild-type HA-FOXP3 or FOXP3 mutants. Twenty-four hours after transfection, total lysates were collected, immunoprecipitated with HA-agarose, followed by immunoblotting with anti-Ac-K or HA-HRP.

(B and C) Acetylation level of wild-type FOXP3 and K250R/K252R mutant in response to the treatment of TGF-β (B), or TGF-β in combination with IL-6 (C). Ten million Jurkat cells were transfected with wild-type or K250R/K252R mutant FOXP3. Forty-eight hours after transfection, cells were stimulated with 50 ng/ml PMA and 1 μM ionomycin, and treated with 5 ng/ml TGF-β or 20 ng/ml IL-6 as indicated for 4 hr. Total lysate were then precipitated with anti-Ac-K and immunoblotted with HA-HRP to check the differential acetylation level of FOXP3 in response to TGF-β and IL-6.

(D) Increased FOXP3 acetylation in response to TGF-β is not affected by FOXP3 K250 and K252 mutations that disrupt FOXP3 dimer formation.

See also Figure S3.

antiparallel mode with residue A229 in the center. A large part of the hydrophobic stretch including residues L214, L222, K226, A229, L233, V237, and L247 were found on the dimer-dimer interface forming the cluster. In addition, residues E211, K215, Q218, E225, E236, S240, Q243, Q244, E248, and K251 contribute to electrostatic stabilization of the dimer-dimer packing in the cluster. The interface accessible surface area between the two adjacent dimers is ~800 Å^2, which is comparable to that of the mFOXP3-ZL coiled-coil dimer itself, representing a transient nature of the clustering interactions.

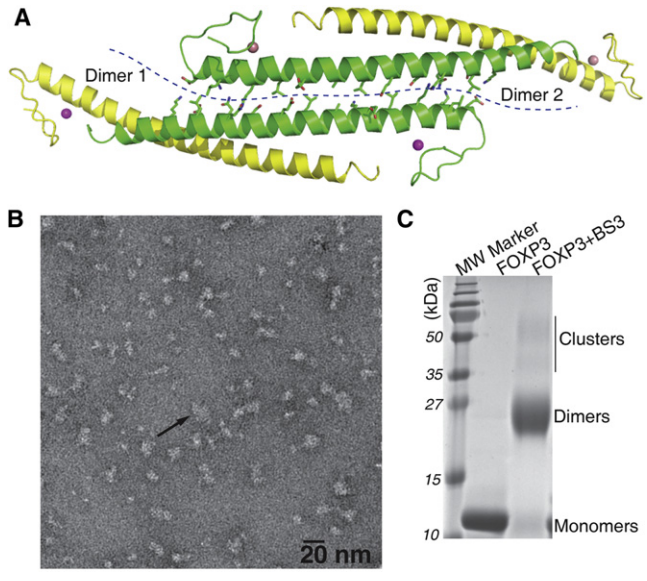

Figure 5. Clustering of FOXP3 Molecules in Crystal and in Solution

(A) A dimer of the FOXP3 coiled-coil dimer observed in mFOXP3 crystals is rendered. The dimer-based packing has a mixed nature of hydrophobic and electrostatic interactions. Residues involved in the dimer-based clustering are rendered in ball-and-stick models and described in the text.

(B) Electron microscopy analysis of FOXP3 molecules in solution. Black arrow represents typical clustering corresponding to a tetrameric association.

(C) Crosslinking analysis indicated that purified recombinant FOXP3 molecules may form high molecular weight clusters in addition to monomer and dimer.

See also Figure S4.

Dimer-based clustering of FOXP3 molecules might be an artifact caused by crystallization. We excluded this possibility by studying both mouse and human FOXP3's clustering propensity in solution, by means of electron microscopy (EM) (Figure 5B) and also by crosslinking analysis using purified recombinant proteins (Figure 5C). While the FOXP3 sample appears as a single band on SDS-PAGE, crosslinking analyses revealed multiple levels of clustering with the dimer being a dominant species and small populations of species of higher order. Negative staining electron microscopy of maltose binding protein-tagged human FOXP3 also revealed high-ordered complexes of FOXP3 (Figure 5B, arrow).

We had previously used gel filtration analyses followed by western blotting to show that full-length FOXP3 was present as a mixture of monomers, dimers, tetramers and high-order oligomers (Li et al., 2007b). Jurkat cells stably transfected with human FOXP3 were treated with histone deacetylase inhibitors. Nuclear fractions were isolated and proteins fractionated by gel filtration (Figure S3). The FOXP3 complex was diminished in overall size from the 670 Kd range leading to a substantial fraction of the ensemble in the range ~158 kDa and less, which tends to be smaller than the size of FOXP3 tetramer. HDACi treatment, via promoting FOXP3 acetylation, may lead to less homoclustering of FOXP3, shifting the FOXP3 oligomerization status toward a dimer as well as loss of certain associated proteins.

Heterotypic Interaction of FOXP3 with FOXP1

As shown by surface plasmon resonance, FOXP1 leucine zipper protein can form heterooligomers with FOXP3 leucine zipper protein (Figure 6A), consistent with our previous observation that FOXP1 colocalizes with FOXP3 at many sites within the nucleus of human CD4$^+$CD25$^+$ T cells. This heteroassociation is disrupted by the IPEX mutation of FOXP3. Moreover, knockdown of endogenous FOXP1 in FOXP3-expressing Jurkat

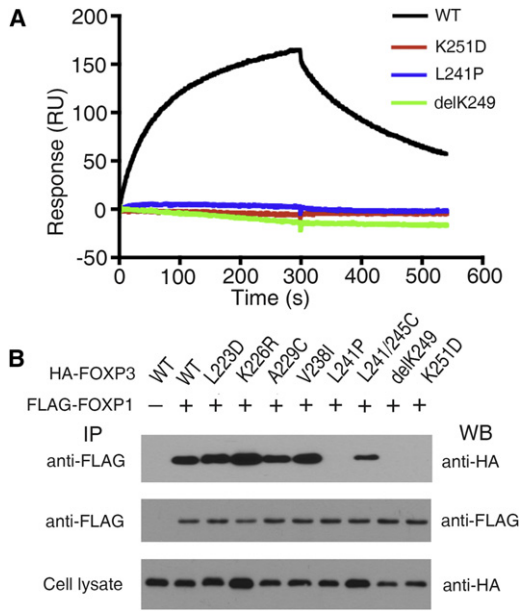

Figure 6. Heteroassociation of FOXP3 with FOXP1

(A) Surface plasmon resonance (SPR) analysis of FOXP1-ZL association with wild-type and mutant FOXP3-ZL using purified recombinant proteins. FOXP1-ZL binds to wild-type FOXP3-ZL, but single mutation L241P or K251D or deletion of K249 abrogated the interaction.

(B) Immunoblotting detection of FOXP1 association with FOXP3 in the context of full-length proteins. The results are consistent with the SPR data.

See also Figure S5.

T cells relieves some FOXP3-mediated repression of IL-2 production (Li et al., 2007b).

Mutations (L241P, delK249, and K251D) that disrupt mFOXP3 homodimeric interactions also impaired FOXP3-FOXP1 heteroassociation (Figures 6A and 6B). On the other hand, mutations that maintained the FOXP3 homoassociation, including V238I on the dimer interface, L223D, K226R, and A229C on the dimer surface, also preserved FOXP3-FOXP1 heteroassociation (Figures 1F and 6B). Thus, a similar interface is likely used to mediate both homo- and heteroassociations of FOXP3 and FOXP1. Furthermore, an arginine residue is found in FOXP1 at the position corresponding to mFOXP3 K251 that is important for the intersubunit hydrogen bonding between Q234 and E248 of FOXP3 (Figures 1B and 1C). Replacement of lysine with arginine at 251 is predicted to constitutively maintain the electrostatic interactions with residues E248 and therefore would stabilize the FOXP1 homodimer.

DISCUSSION

FOXP3 is a transcriptional regulator acting as a component of a dynamic multisubunit complex involved in histone modification and chromatin remodeling after TCR stimulation (Li et al., 2006, 2007a). Acetylated forms of FOXP3 are regulated and can be induced by T cell receptor and TGF-β signals, while the levels of acetylated FOXP3 are diminished by inflammatory signals. In vivo we had previously found that a pool of acetylated forms of FOXP3 are loosely associated with chromatin (Samanta et al., 2008). Here we define the atomic features of the dimeric assembly of the FOXP3 coiled coil and correlates structure with disease mutations and functional regulation.

Coiled- Coil-Mediated Dimer Is a Regulatory Structure Important for FOXP3 Function

Our studies revealed substantial structural instability and irregularities of the FOXP3 leucine zipper. For example, a high proportion of polar residues were found at the core packing (Figure 1A), and sizable holes were formed on the dimeric interface passing through the hydrophobic core (Figures 1D and 1E). Moreover, statistical analyses of accessible surface and empty space of the dimeric interface indicated transient and dynamic features of the FOXP3 homodimer.

The intact leucine zipper is required for DNA binding through the FOXP3 forkhead domain (Koh et al., 2009; Li et al., 2007b). Our current study explains at the atomic level how disruption of homodimerization abrogates FOXP3 function in vitro and in vivo. Based on its antiparallel conformation, the FOXP3 coiled coil also plays a role in coordinating DNA binding with coregulator recruitment (Figure S5). Since the FOXP3 coiled coil is not tightly assembled, we envision it to exist in a flexible conformation able to undergo tightening to form a stable dimer for high-affinity DNA binding upon differential posttranslational modifications, or loosening to break apart for heteroassociation with FOXP1. Thus, the plasticity of the FOXP3 coiled coil could provide a regulatory basis, through which the oligomerization status and stability of FOXP3 is controlled in a cell context "signaling"-dependent manner.

Atomic Explanation of IPEX Disease-Causing Mutations on FOXP3 Structure

Due to the 2-fold symmetrical conformation of the FOXP3 coiled coil, mutation of any single residue will affect two sites of the dimer, which therefore will enhance the biological consequence of the mutational changes caused by diseases. The structure of mFOXP3-ZL revealed that deletion of a single residue in the coiled coil would break the heptad-based symmetrical packing, resulting in mismatch of residues on the dimeric interface (Figure 1A). A network of interactions involving Q234, E242, E248, K249, and K251 has been identified to stabilize FOXP3 homodimerization (Figures 1C, 1D, 7A, and 7B). Notably K251 of α helix 1 forms an intrasubunit hydrogen bond with E248, holding E248 to further form an intersubunit hydrogen bond with Q234 of α helix 2 (Note that α helices 1 and 2 form the mFOXP3 coiled coil). In addition, K249 of α helix 1 directly forms an intersubunit salt bridge with E242 of α helix 2. These observations readily explain several reported deletions found in an IPEX patient (human FOXP3 DelK250 and DelE251, equivalent to mFOXP3 K249 and E250) (Chatila et al., 2000; Wildin et al., 2002). Deletions of these key residues may either directly break the E242-K249 intersubunit salt bridge, or cause a positional shift and strain or break the Q234-E248 intersubunit hydrogen bond through conformational "releasing" of E248 (Figures 7A and 7B). As a result, these deletion mutants will disrupt the homodimerization of FOXP3 (Figure 1F) and lead to impaired ability to repress transcription (Figures 2 and 3) (Li et al., 2007b; Ziegler, 2006).

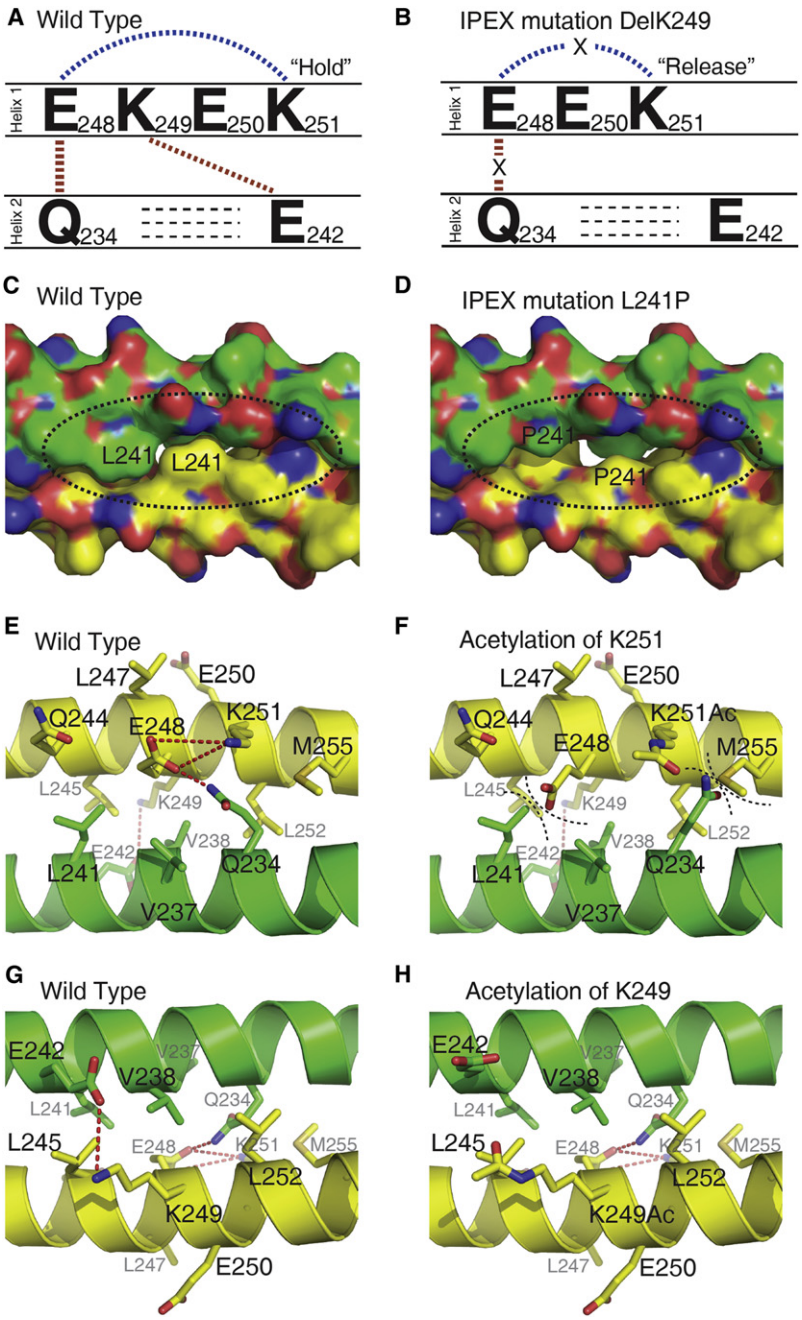

Figure 7. Structural Models of IPEX Mutations and Lysine Acetylation of FOXP3

(A, E, G) FOXP3 lysine 251 posed the side chain of core residue E248, which then forms intersubunit hydrogen bond with the side chain of core residue Q234. On the opposite side of the coiled coil, K249 forms intersubunit salt bridge with E242.

(B) Deletion of K249 found in some IPEX patients not only breaks its direct interaction with E242 from the opposing α helix, but also alters the positioning of K251 relative to E248, thus releases the side chain of E248, which then breaks the intersubunit bonding between E248 and Q234.

(C and D) IPEX mutation L241P generates a solvent accessible groove on the dimeric interface of FOXP3 coiled coil, making the packing hole around L241 much larger compared with wild-type.

(E and F) The modeled acetylated form of lysine 251 (K251-Ac) neutralizes the positive charge of lysine, disrupting its interaction with the side chain of E248. Conformational variation of E248 may therefore impair its interaction with Q234, releasing the side chain of Q234. The resultant close contacts between L241 and E248, Q234, and M255 may further destabilize the FOXP3 homodimerization.

(G and H) The modeled acetylated form of lysine 249 (K249-Ac) is predicted to directly disrupt the intersubunit salt bridge between K249 and E242.

Human FOXP3 L242P (equivalent to mFOXP3 L241P) is a mutation found in an IPEX patient (Gambineri et al., 2008; Zuo et al., 2007). It is worth noting that L241 of mFOXP3 is located at the core position of the coiled-coil center, where the 2-fold axis passes along (Figures 1E and 7C). Variation at this site is expected to directly alter the coiled-coil conformation and stability, and therefore affect the regulatory function of the FOXP3 homodimer. In the wild-type mFOXP3 coiled coil, there is a hole near L241 and L245, indicative of a flexible nature of the homodimer. Substitution of L241 with a proline may not only distort the helical conformation, but also make the hole near L241 and L245 even larger. The smaller side chain of proline would leave more empty space in the core packing, generating a groove (Figure 7D). One consequence of this packing is that water molecules may pass through the dimer interface causing instability (Figure 1F).

Modulation of FOXP3 Structure and Function by Acetylation

Mass spectrometry and biochemical studies identified and confirmed human FOXP3 K250 and K252, i.e., K249 and K251 of mFOXP3, as acetylation sites in the coiled coil. Our current structure indicates that acetylation sites K251 and K249 are electrostatically involved in the interface network critical for FOXP3 dimerization (Figure 7). p300 appears to target these sites (K250 and K252 in human FOXP3) to acetylate FOXP3. Mutational analysis of these sites (K249-251D/Q/R) indicates that acetylation appears to be important for regulating the FOXP3 dimer formation and, suppressive activity. Recently van Loosdregt et al. suggested that p300 could promote the acetylation level of wild-type human FOXP3 (van Loosdregt et al., 2010).

Acetylation of K249 and/or K251 appears to modify the same set of dimeric interactions affected by IPEX mutations. As discussed above, the key intersubunit hydrogen bond between Q234 and E248 is dependent on the unique conformation of the E248 side chain that is held by the side chain of K251 (Figures 7A and 7E). Acetylation of K251 will neutralize the positive charges of its side chain and, therefore, decrease its interaction

with E248. In turn, the release of the E248 side chain will break the intersubunit hydrogen pairing between Q234 and E248, further releasing the side chain of Q234 (Figures 7B and 7F). As a result, the steric tension caused by E248-L241 and Q234-M255 may further relax/destabilize the FOXP3 homodimerization (Figures 3A and 7F). Similarly, acetylation of K249 will directly decrease its intersubunit pairing with E242, as observed for the IPEX mutant DelK249 (Figures 7G and 7H). Taken together, acetylation of K251 and/or K249 may impose structural and functional modifications on the FOXP3 molecules similar to the IPEX mutations, but in a regulated manner.

Considering the irregularity and instability of the FOXP3 coiled coil and the potential acetylation sites, we speculate that acetylation/deacetylation of the coiled-coil regulatory structure possibly by TIP60 and HDAC7/HDAC9 may reversibly shift the FOXP3 molecule ensemble between nonstable homodimers and stable homodimers and heterodimers. From the in vitro experiment (Figure 3A), we observed that the flexibility of FOXP3 to have acetylation at K251 is optimal for the formation of dimer. When K251 is replaced by an acetylation-mimicking residue K251Q or the negatively charged K251D, formation of dimer is reduced. A positively charged residue, however, is preferred at K249 for dimer formation, as no effect is observed for the K249R mutant.

Acetylation of FOXP3 by discrete signals may have contrasting effects on different lysines. FOXP3 acetylation has been shown to increase FOXP3 in vivo protein levels by lessening degradation (van Loosdregt et al., 2010). We found that acetylation at FOXP3 K249/251 may downregulate its suppressive activity by causing dimer relaxation. However, TGF-β does not promote FOXP3 acetylation at K249 and K251 that are dominantly targeted by p300 (Figure 4). These data indicate that lysine modifications of K249 and K251 lead to "mechanistic adjustments" of the dimeric complex of FOXP3 proteins but are not relevant to the type of the functional change induced by TGF-β and the acetylation pattern that cytokine stimulates. In fact other lysine residues undergo specific acetylation encouraged by TGF-β, and we propose that those modifications alone or in combination with other posttranslational modifications are responsible for increased affinity to chromatin and enhanced suppressive behavior.

Regulation of FOXP3 Complex through Homoclustering and Heterotypic Interactions

Our crystallographic study identified a hydrophobic stretch on the molecular surface of the FOXP3 dimer that may promote homoclustering (Figure S4). Further solution studies confirmed that FOXP3 may indeed form high molecular weight clusters (Li et al., 2007b) (Figures 5B and 5C). FOXP3 homoclustering is diminished by mutations of the residues on the homodimer interface that disrupts homodimerization. Homoclustering of human FOXP3 itself can be modified by HDAC inhibitor treatment (Figure S3), suggesting a physiological role in FOXP3-mediated suppression of higher order complex formation.

FOXP3 can interact with FOXP1 through the leucine zipper domain. Specifically, FOXP3 forms heterodimers with FOXP1 likely via an interface similar to that involved in FOXP3 homodimer formation (Figure 3). FOXP1-FOXP3 heteroassociations may compete with FOXP3 homodimerization and/or homoclustering and the dynamic balance of discrete forms of FOXP3 complexes may directly affect its repressor activity (Figure S5).

Previously we reported that the FOXP3 coiled coil may associate with HEAT-SHOCK 70-KD PROTEIN 5 (HSPA5/BiP/GRP78), as well as with the linker histone H1.5 (Li et al., 2007c). Lately *Su* and colleagues found that histone H1.5 and FOXP3 interact with each other to cooperatively repress IL-2 transcription in human T cells (Mackey-Cushman et al., 2011). IPEX mutations found in the leucine zipper domain that disrupted FOXP3 homodimerization abolished FOXP3 binding to histone H1.5, indicating that heteroassociation between H1.5 and FOXP3 is dependent on the coiled-coil-mediated homodimerization of FOXP3.

Conclusions

We have resolved certain of the atomic features of homo- and heteroassociation of FOXP3. Clearly, it is the homomeric FOXP3 dimer assembled with other coregulators and in combination with discrete posttranslational modifications of discrete FOXP3 residues, that ultimately alters the conformation and activity of the FOXP3 protein complex to modulate Treg function. Appreciation of the structural features of the FOXP3 coiled coil explains the actions of many of the mutations involved in human diseases. FOXP3 are acetylated at several lysines, K8, K382, and K389 (Tao et al., 2007; Samanta et al., 2008) and K249 and K251 (this work), K262 and K267 (M.I.G., unpublished data). A recent study on acetylation sites of FOXP3, have found a role for K31 and K267 in the regulation of the suppressive activity of regulatory T cells (Kwon et al., 2012). A feature of the Kwon et al. study that extends our thesis is that K31 and K262 may be targets of Sirtuin deacetylation. It is also worth noting that discrete acetylation sites could be differentially modified by different HATs depending on distinct stimulations and cellular context, which may result in differential control of the FOXP3 molecules. Our current work has focused on the acetylation of only K249 and K251, and described an atomic explanation for FOXP3 acetylation at K249/251, which alters the stability of the dimeric complex of FOXP3 proteins. Knowledge of the structure of enzymatic modifications on FOXP3 may also be useful for modifying immunity to HER2 tumors in which FOXP3+ cells are relevant (M.I.G, unpublished data).

EXPERIMENTAL PROCEDURES

The experimental procedures used in this manuscript are briefly described as below. Supplemental Information contains a complete description with more details.

Protein Expression and Purification

Proteins used in this study were all expressed in *Escherichia coli* and purified by Strep-Tactin affinity column followed by gel filtration. All the mutants used for immunoprecipitation were made based on the full-length FOXP3.

Crystallographic Studies

Crystals were obtained by mixing equal volumes of sample solution (20 mM HEPES [pH 7.3], 1 mM TCEP, 0.5% Chaps, and 100 mM NaCl) and well buffer (100 mM NaAC [pH 4.5] and ~1.8 M $MgSO_4$) for hanging drop. Crystals were croprotected by adding 30% glycerol. Native and multiwavelength anomalous

dispersion (selenium) data were collected at BL17U beam line, Shanghai Synchrotron Radiation Facility (Table S1). Structure determination and modeling were carried out with standard procedures as previously published (Brown et al., 2005).

Cell Culture and Immunoprecipitation

293T cells transfected with HA-FOXP3 or FLAG-FOXP3 were prepared for immunoprecipitation with anti-FLAG antibody, followed by western blot analysis using anti-HA antibody. The expression levels of the exogenous FOXP3 proteins were also examined by western blot using anti-HA or anti-FLAG antibody.

Spectroscopy-LC-MS

10 μl of the IP pull-down solution was diluted with the running buffer of a SDS-PAGE gel (10% Tris-HEPES-SDS gel) for separation. The gel bands of interest were identified and isolated for trypsin digestion. After MS data collection, protein identification was achieved through computer aided searching of the Swiss-Prot human protein database using SEQUEST.

Biacore Analysis

Binding experiments were performed with the surface plasmon resonance-based biosensor instrument Biacore 2000 (Biacore), at 25°C, essentially as described before (Berezov et al., 2002). Immobilization of FOXP3 on the sensor surface was performed following the standard amine coupling procedure according to manufacturer's instructions.

Suppression Assay

In vitro assays of Treg function were performed using MACS-purified CFSE-labeled $CD4^+$ $CD25^-$ T cells from normal C57BL/6 mice as effector cells, and normal $CD4^+$ $CD25^+$ Tregs or Foxp3-transduced cells as regulatory cells, with flow cytometric assessment of CFSE dilution at 72 hr and data analysis using Flowjo 4.2 software (TreeStar) (Tao et al., 2007). The ability of Tregs to suppress the homeostatic proliferation of $Thy1.1^+$ B6 $CD4^+CD25^-$ T cells was assessed by the coinjection, i.v., of 10^6 cells of each type into $B6/RAG2^{-/-}$ mice (Tao et al., 2007).

ACCESSION NUMBERS

The atomic coordinates and structure factors have been deposited in the Protein Data Bank, http://www.pdb.org (PDB ID 3HSX).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, and one table and can be found with this article online at doi:10.1016/j.celrep.2012.04.012.

LICENSING INFORMATION

ACKNOWLEDGMENTS

This work was supported by the 973 program of the Ministry of Science and Technology of China (2010CB529701, 2012CB910204), the National Institutes of Health (NIH; 5P01AI073489 and 2R01CA055306 to M.I.G.), the Breast Cancer Research Foundation (M.I.G.), the National Natural Science Foundation of China (NSFC10979005, NSFC30972702, NIH-NSFC collaborative grant 81161120417), and a Science and Technology Commission of Shanghai Municipality grant (SMCST09JC1416100). B.L.K. acknowledges NIH GM 15847 and contribution no. 965 from the Barnett Institute for support of this work.

Received: February 8, 2012
Revised: March 20, 2012
Accepted: April 30, 2012
Published online: June 14, 2012

REFERENCES

Bandukwala, H.S., Wu, Y., Feuerer, M., Chen, Y., Barboza, B., Ghosh, S., Stroud, J.C., Benoist, C., Mathis, D., Rao, A., and Chen, L. (2011). Structure of a domain-swapped FOXP3 dimer on DNA and its function in regulatory T cells. Immunity *34*, 479–491.

Bennett, C.L., Christie, J., Ramsdell, F., Brunkow, M.E., Ferguson, P.J., Whitesell, L., Kelly, T.E., Saulsbury, F.T., Chance, P.F., and Ochs, H.D. (2001). The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat. Genet. *27*, 20–21.

Berezov, A., Chen, J., Liu, Q., Zhang, H.T., Greene, M.I., and Murali, R. (2002). Disabling receptor ensembles with rationally designed interface peptidomimetics. J. Biol. Chem. *277*, 28330–28339.

Bettelli, E., Dastrange, M., and Oukka, M. (2005). Foxp3 interacts with nuclear factor of activated T cells and NF-kappa B to repress cytokine gene expression and effector functions of T helper cells. Proc. Natl. Acad. Sci. USA *102*, 5138–5143.

Brown, J.H., Zhou, Z., Reshetnikova, L., Robinson, H., Yammani, R.D., Tobacman, L.S., and Cohen, C. (2005). Structure of the mid-region of tropomyosin: bending and binding sites for actin. Proc. Natl. Acad. Sci. USA *102*, 18878–18883.

Brunkow, M.E., Jeffery, E.W., Hjerrild, K.A., Paeper, B., Clark, L.B., Yasayko, S.A., Wilkinson, J.E., Galas, D., Ziegler, S.F., and Ramsdell, F. (2001). Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat. Genet. *27*, 68–73.

Chae, W.J., Henegariu, O., Lee, S.K., and Bothwell, A.L. (2006). The mutant leucine-zipper domain impairs both dimerization and suppressive function of Foxp3 in T cells. Proc. Natl. Acad. Sci. USA *103*, 9631–9636.

Chatila, T.A., Blaeser, F., Ho, N., Lederman, H.M., Voulgaropoulos, C., Helms, C., and Bowcock, A.M. (2000). JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. J. Clin. Invest. *106*, R75–R81.

Chen, C., Rowell, E.A., Thomas, R.M., Hancock, W.W., and Wells, A.D. (2006). Transcriptional regulation by Foxp3 is associated with direct promoter occupancy and modulation of histone acetylation. J. Biol. Chem. *281*, 36828–36834.

Fontenot, J.D., Gavin, M.A., and Rudensky, A.Y. (2003). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat. Immunol. *4*, 330–336.

Gambineri, E., Torgerson, T.R., and Ochs, H.D. (2003). Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance (IPEX), a syndrome of systemic autoimmunity caused by mutations of FOXP3, a critical regulator of T-cell homeostasis. Curr. Opin. Rheumatol. *15*, 430–435.

Gambineri, E., Perroni, L., Passerini, L., Bianchi, L., Doglioni, C., Meschi, F., Bonfanti, R., Sznajer, Y., Tommasini, A., Lawitschka, A., et al. (2008). Clinical and molecular profile of a new series of patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome: inconsistent correlation between forkhead box protein 3 expression and disease severity. J. Allergy Clin. Immunol. *122*, 1105–1112, e1.

Godfrey, V.L., Wilkinson, J.E., and Russell, L.B. (1991). X-linked lymphoreticular disease in the scurfy (sf) mutant mouse. Am. J. Pathol. *138*, 1379–1387.

Hori, S., Nomura, T., and Sakaguchi, S. (2003). Control of regulatory T cell development by the transcription factor Foxp3. Science *299*, 1057–1061.

Khattri, R., Cox, T., Yasayko, S.A., and Ramsdell, F. (2003). An essential role for Scurfin in CD4+CD25+ T regulatory cells. Nat. Immunol. *4*, 337–342.

Koh, K.P., Sundrud, M.S., and Rao, A. (2009). Domain requirements and sequence specificity of DNA binding for the forkhead transcription factor FOXP3. PLoS ONE *4*, e8109.

Kwon, H.S., Lim, H.W., Wu, J., Schnölzer, M., Verdin, E., and Ott, M. (2012). Three novel acetylation sites in the Foxp3 transcription factor regulate the suppressive activity of regulatory T cells. J. Immunol. *188*, 2712–2721.

Li, B., Samanta, A., Song, X., Furuuchi, K., Iacono, K.T., Kennedy, S., Katsumata, M., Saouaf, S.J., and Greene, M.I. (2006). FOXP3 ensembles in T-cell regulation. Immunol. Rev. *212*, 99–113.

Li, B., Samanta, A., Song, X., Iacono, K.T., Bembas, K., Tao, R., Basu, S., Riley, J.L., Hancock, W.W., Shen, Y., et al. (2007a). FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. Proc. Natl. Acad. Sci. USA *104*, 4571–4576.

Li, B., Samanta, A., Song, X., Iacono, K.T., Brennan, P., Chatila, T.A., Roncador, G., Banham, A.H., Riley, J.L., Wang, Q., et al. (2007b). FOXP3 is a homo-oligomer and a component of a supramolecular regulatory complex disabled in the human XLAAD/IPEX autoimmune disease. Int. Immunol. *19*, 825–835.

Li, B., Saouaf, S.J., Samanta, A., Shen, Y., Hancock, W.W., and Greene, M.I. (2007c). Biochemistry and therapeutic implications of mechanisms involved in FOXP3 activity in immune suppression. Curr. Opin. Immunol. *19*, 583–588.

Lin, W., Haribhai, D., Relland, L.M., Truong, N., Carlson, M.R., Williams, C.B., and Chatila, T.A. (2007). Regulatory T cell development in the absence of functional Foxp3. Nat. Immunol. *8*, 359–368.

Lopes, J.E., Torgerson, T.R., Schubert, L.A., Anover, S.D., Ocheltree, E.L., Ochs, H.D., and Ziegler, S.F. (2006). Analysis of FOXP3 reveals multiple domains required for its function as a transcriptional repressor. J. Immunol. *177*, 3133–3142.

Mackey-Cushman, S.L., Gao, J., Holmes, D.A., Nunoya, J.I., Wang, R., Unutmaz, D., and Su, L. (2011). FoxP3 interacts with linker histone H1.5 to modulate gene expression and program Treg cell activity. Genes Immun. *12*, 559–567.

Ono, M., Yaguchi, H., Ohkura, N., Kitabayashi, I., Nagamura, Y., Nomura, T., Miyachi, Y., Tsukada, T., and Sakaguchi, S. (2007). Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1. Nature *446*, 685–689.

Pavletich, N.P., and Pabo, C.O. (1993). Crystal structure of a five-finger GLI-DNA complex: new perspectives on zinc fingers. Science *261*, 1701–1707.

Qin, S., Cobbold, S.P., Pope, H., Elliott, J., Kioussis, D., Davies, J., and Waldmann, H. (1993). "Infectious" transplantation tolerance. Science *259*, 974–977.

Reynolds, C., Damerell, D., and Jones, S. (2009). ProtorP: a protein-protein interaction analysis server. Bioinformatics *25*, 413–414.

Rudensky, A.Y., and Campbell, D.J. (2006). In vivo sites and cellular mechanisms of T reg cell-mediated suppression. J. Exp. Med. *203*, 489–492.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., and Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J. Immunol. *155*, 1151–1164.

Sakaguchi, S., Ono, M., Setoguchi, R., Yagi, H., Hori, S., Fehervari, Z., Shimizu, J., Takahashi, T., and Nomura, T. (2006). Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. Immunol. Rev. *212*, 8–27.

Samanta, A., Li, B., Song, X., Bembas, K., Zhang, G., Katsumata, M., Saouaf, S.J., Wang, Q., Hancock, W.W., Shen, Y., and Greene, M.I. (2008). TGF-beta and IL-6 signals modulate chromatin binding and promoter occupancy by acetylated FOXP3. Proc. Natl. Acad. Sci. USA *105*, 14023–14027.

Schwartz, R.H. (2005). Natural regulatory T cells and self-tolerance. Nat. Immunol. *6*, 327–330.

Strelkov, S.V., and Burkhard, P. (2002). Analysis of α-helical coiled coils with the program TWISTER reveals a structural mechanism for stutter compensation. J. Struct. Biol. *137*, 54–64.

Stroud, J.C., Wu, Y., Bates, D.L., Han, A., Nowick, K., Paabo, S., Tong, H., and Chen, L. (2006). Structure of the forkhead domain of FOXP2 bound to DNA. Structure *14*, 159–166.

Tao, R., de Zoeten, E.F., Ozkaynak, E., Chen, C., Wang, L., Porrett, P.M., Li, B., Turka, L.A., Olson, E.N., Greene, M.I., et al. (2007). Deacetylase inhibition promotes the generation and function of regulatory T cells. Nat. Med. *13*, 1299–1307.

van Loosdregt, J., Vercoulen, Y., Guichelaar, T., Gent, Y.Y., Beekman, J.M., van Beekum, O., Brenkman, A.B., Hijnen, D.J., Mutis, T., Kalkhoven, E., et al. (2010). Regulation of Treg functionality by acetylation-mediated Foxp3 protein stabilization. Blood *115*, 965–974.

Wang, B., Lin, D., Li, C., and Tucker, P. (2003). Multiple domains define the expression and regulatory properties of Foxp1 forkhead transcriptional repressors. J. Biol. Chem. *278*, 24259–24268.

Wildin, R.S., Ramsdell, F., Peake, J., Faravelli, F., Casanova, J.L., Buist, N., Levy-Lahad, E., Mazzella, M., Goulet, O., Perroni, L., et al. (2001). X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. Nat. Genet. *27*, 18–20.

Wildin, R.S., Smyk-Pearson, S., and Filipovich, A.H. (2002). Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. J. Med. Genet. *39*, 537–545.

Wu, Y., Borde, M., Heissmeyer, V., Feuerer, M., Lapan, A.D., Stroud, J.C., Bates, D.L., Guo, L., Han, A., Ziegler, S.F., et al. (2006). FOXP3 controls regulatory T cell function through cooperation with NFAT. Cell *126*, 375–387.

Xiao, Y., Li, B., Zhou, Z., Hancock, W.W., Zhang, H., and Greene, M.I. (2010). Histone acetyltransferase mediated regulation of FOXP3 acetylation and Treg function. Curr. Opin. Immunol. *22*, 583–591.

Ziegler, S.F. (2006). FOXP3: of mice and men. Annu. Rev. Immunol. *24*, 209–226.

Zuo, T., Wang, L., Morrison, C., Chang, X., Zhang, H., Li, W., Liu, Y., Wang, Y., Liu, X., Chan, M.W., et al. (2007). FOXP3 is an X-linked breast cancer suppressor gene and an important repressor of the HER-2/ErbB2 oncogene. Cell *129*, 1275–1286.